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Effects of bucillamine and N-acetyl-L-cysteine on cytokine production and collagen-induced arthritis (CIA)

F. TSUJI, Y. MIYAKE, H. AONO, Y. KAWASHIMA & S. MITA *Discovery Research Division, Santen Pharmaceutical Co., Ltd, Osaka, Japan*

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SUMMARY

We investigated the effects of bucillamine and N-acetyl-L-cysteine (NAC) on cytokine production and CIA. Bucillamine and NAC inhibited NF-κB activation and tumour necrosis factor-alpha (TNF-α) mRNA expression in human monocytic leukaemia cell line THP-1, and cytokine production from monocyte cell lines at concentrations $>10^{-3}$ M. They also inhibited cytokine production and CIA in mice at a dose of 500 mg/kg. These results suggest that NF-κB inhibitors such as bucillamine and NAC may inhibit cytokine-related diseases, including arthritis.

Keywords bucillamine N-acetyl-L-cysteine NF-κB cytokine arthritis

INTRODUCTION

Cytokines are peptide hormones that regulate a wide variety of immune and inflammatory processes [1,2]. As a consequence of their central roles in the regulation of immunological and inflammatory processes, cytokines are pivotal mediators of autoimmune, inflammatory and collagen-vascular diseases. Rheumatoid arthritis (RA) is a chronic and progressive inflammatory process with systemic immunological abnormalities leading to synovial hyperplasia and joint destruction. The inflamed synovium is infiltrated by lymphocytes and monocytes, which reinforce the underlying immunological mechanism in this disease process [3–6]. Although the pathogenesis of RA remains unknown, cytokines and cell adhesion molecules (CAM) have been suggested to be actively involved in rheumatoid inflammation. These cytokines include tumour necrosis factor-alpha (TNF-α), IL-1, IL-6, IL-8, interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [6–8]. Among these cytokines, TNF-α and IL-1 have been studied most extensively because of their actions in inducing the expression of other cytokines and CAM. This has been confirmed by clinical trials using anti-TNF-α MoAb and IL-1 antagonists in the treatment of RA synovitis [9,10]. Furthermore, it is well established that TNF-α and IL-1 stimulate gene expression of these cytokines and CAM through a signal transduction pathway leading to NF-κB activation [11–16].

NF-κB is an inducible cellular transcription factor present in the primordial mesenchymal cell lineage including lymphocytes, macrophages and fibroblasts [11,16]. NF-κB regulates a wide variety of cellular genes including those associated with RA. Although NF-κB is by no means the sole determinant for the inducible expression of these genes, it has been shown to play a significant role in inducing their expression [11–16]. It has been reported that an IκB kinase is involved in NF-κB activation by directly phosphorylating IκB [17,18]. Although specific inhibitors of kinases involved in the NF-κB activation cascade have yet to be identified, antioxidants such as N-acetyl-L-cysteine (NAC) [19,20] are known to block the NF-κB cascade.

The anti-inflammatory effects of steroids, retinoids and a variety of anti-rheumatic drugs take place by mechanisms that converge on a limited number of transcription factors, most notably the pro-inflammatory transcription factors AP-1 and NF-κB [21]. Bucillamine (N-(mercapto-2-methylpropionyl)-L-cysteine), a synthetic sulfhydryl (SH) compound like NAC developed as a disease-modifying anti-rheumatic drug (DMARD) for the treatment of RA, has shown clinical efficacy in RA and related arthritides [22].

In the present study we investigate the role of NF-κB in cytokine production and RA using bucillamine and NAC to block the NF-κB activation pathway.

Correspondence: Fumio Tsuji, Discovery Research Division, Santen Pharmaceutical Co., Ltd, 3-9-19 Shimoshinjo, Higashiyodogawa-ku, Osaka 533-8651, Japan.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS; *Escherichia coli* 055; B5; Difco, Detroit, MI), bovine type II collagen (CII; Cosmobio, Tokyo, Japan), Freund's complete adjuvant (FCA; Difco), RPMI1640 (Gibco, Rockville, MD), fetal calf serum (FCS; Gibco), HEPES (Nacalai Tasque, Tokyo, Japan), Nonidet P-40 (Nacalai Tasque), KCl (Wako, Tokyo, Japan), $MgCl_2$ (Wako), PMSF (Wako), aprotinin (Wako), dithiothreitol (DTT; Sigma, St Louis, MO), EDTA-2Na (Dojindo, Tokyo, Japan) and NAC (Sigma) were purchased from the sources shown. Bucillamine (N-(mercapto-2-methylpropionyl)-L-cysteine) was synthesized by the Central Research Laboratories of Santen Pharmaceutical Co., Ltd.

Cell line and cell culture

Human monocytic leukaemia cell line THP-1 and mouse monocytic leukaemia cell line J774.1 were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI1640 supplemented with 10% FCS and 50 μM 2-mercaptoethanol.

Nuclear extracts and electrophoretic mobility shift assay

The cells were cultured in the presence or absence of drugs with 2 μg/ml of LPS for 1 h and nuclear extracts were prepared as described by Molitor *et al.* [23] with minor modifications. Briefly, THP-1 cells (1×10^6 cells) were harvested and incubated with buffer A (10 mM HEPES pH 7·8, 10 mM KCl, 2·0 mM $MgCl_2$, 1·0 mM DTT, 0·1 mM EDTA, 0·1 mM PMSF, 100 U/ml aprotinin) for 15 min at 4°C. Nonidet P40 solution (final concentration 0·6%) was then added and the cells were centrifuged for 30 s at 12 000 ***g***. Pelleted nuclei were suspended with buffer B (50 mM HEPES pH 7·8, 50 mM KCl, 300 mM NaCl, 1·0 mM DTT, 0·1 mM EDTA, 0·1 mM PMSF, 10% glycerol, 100 U/ml aprotinin) and centrifuged for 5 min at 12 000 ***g***. The protein concentration of the nuclear extract was determined by Bradford assay. NF-κB activation was examined by electrophoretic mobility shift assay (EMSA) for ^{32}P-labelled NF-κB oligonucleotide binding. An oligonucleotide containing the NF-κB consensus sequence (5′-AGTTGAGGG-GACTTTCCCAGGC-3′) was used with a gel shift assay kit (Promega, Madison, WI).

Cytokine production

THP-1 cells, suspended at a concentration of 2×10^6 cells/ml in RPMI1640, were incubated at 37°C for 2 h in the presence or absence of drugs with 2 μg/ml of LPS. TNF-α, IL-1β and IL-8 production from THP-1 cells was determined by ELISA using a commercial kit (Amersham, Aylesbury, UK). Similarly, J774.1 cells, suspended at a concentration of 2×10^6 cells/ml in RPMI1640, were also incubated at 37°C for 2 h in the presence or absence of drugs with 2 μg/ml of LPS. TNF-α and IL-6 production from J774.1 cells was determined by ELISA (Amersham).

Extraction of RNA

Total RNA was isolated from THP-1 cells (2×10^6 cells) using a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) 1 h after LPS stimulation. The extracted RNA was quantified and aliquots of 0·1 μg were used to make cDNA.

cDNA synthesis and polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using a commercial kit (Takara, Otsu, Japan). The following conditions were used: denaturation, 94°C for 30 s; annealing, 60°C for 30 s; extension, 72°C for 90 s. The reaction was initiated by adding two units of Taq DNA polymerase, after which 25 PCR cycles were carried out using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The primers used were GAG-TGACAAGCCTGTAGCCCATGTTGTAGCA (sense) and GCA-ATGATCCCAAAGTAGACCTGCCCAGACT (anti-sense) for TNF-α, and TGAAGGTCGGAGTCAACGGATTTGGT (sense) and CATGTGGGCCATGAGGTCCACCAC (anti-sense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA).

TNF-α and IL-6 production in mice

C3H/HeN mice (Japan SLC Inc., Hamamatsu, Japan) at the age of 6–7 weeks were injected intraperitoneally with 0·2 mg/kg LPS suspension. Plasma samples were collected from each animal 1 h after LPS injection for analysis of the amount of TNF-α and 2 h after LPS injection for analysis of the amount of IL-6.

CII-induced arthritis

CII-induced arthritis was brought about in DBA/1 J mice (Charles River Japan, Yokohama, Japan) essentially according to the previously described method [24]. Mice were injected intradermally into the base of the tail with 200 μg of bovine CII emulsified in FCA. Three weeks after the initial injection, a booster injection of 200 μg of bovine CII emulsified in FCA was performed intradermally into the base of the tail. Evaluation of clinical arthritis activity was carried out every 3 days from the second immunization for 27 days and its severity in the metacarpophalangeal wrist, metatarsophalangeal and ankle joints was scored as 0 = no arthritis, 1 = small degree of arthritis, 2 = light swelling, 3 = medium swelling, 4 = severe swelling and non-weight-bearing. The arthritic score was the sum of the scores of all joints involved. At the end of the experimental period (27 days), radiographic assessment of skeletal changes was performed using a Sofron x-ray apparatus (Soken, Tokyo, Japan). Bone changes were graded on a scale of 0–2: 0 = negative, 1 = mild, 2 = severe. The final bone changes score was considered to be the sum of the scores of pelvic limbs. In addition, plasma samples were collected from each animal for analysis of IgG anti-CII antibody levels and the amounts of TNF-α and IL-6.

Determination of IgG anti-CII antibody levels and the amounts of TNF-α and IL-6 in plasma

Anti-CII antibody levels were measured by ELISA using a mouse IgG anti-CII antibody assay kit (Chondrex, Seattle, WA). The amounts of TNF-α and IL-6 were also measured by ELISA using a commercial kit (Amersham).

Administration of drugs

To investigate the effects of bucillamine and NAC on TNF-α and IL-6 production in mice, drugs in 1% methyl cellulose solution (vehicle) were given orally just before LPS injection. And to evaluate the effects of bucillamine and NAC on CII-induced arthritis, drugs (500 mg/kg per day) in the vehicle were given orally for 27 days from the second immunization. Animals in the control group were given the vehicle only orally.

Statistical analysis

Results were statistically evaluated by Dunnett's multiple comparison test or Wilcoxon test (StatLight; Yukms Corp., Tokyo, Japan).

RESULTS

Induction of NF-κB binding in THP-1 cells

The induction of NF-κB binding with LPS was analysed by EMSA. Induction of THP-1 with 2 μg/ml LPS resulted in the appearance of shifted NF-κB bands, but these bands did not appear in the absence of LPS (Fig. 1). Control experiments indicated that these bands were eliminated by a 50-fold excess of unlabelled NF-κB probe,

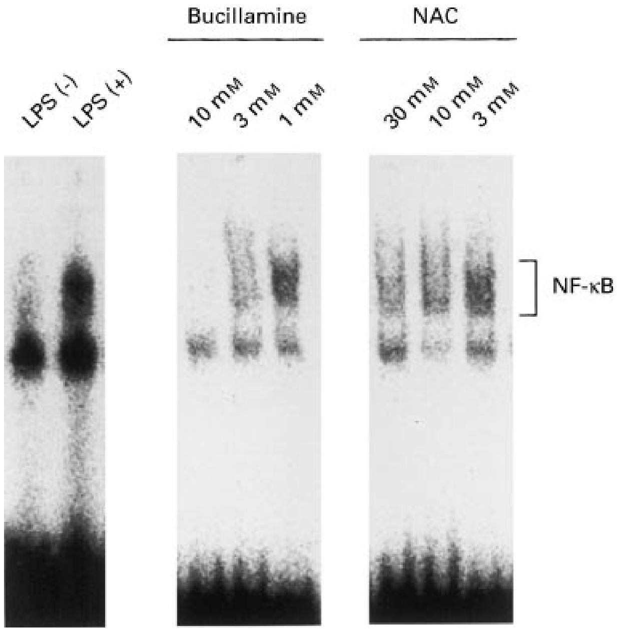

Fig. 1. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on binding of NF-κB to the probe in lipopolysaccharide (LPS)-stimulated THP-1 cells. LPS-stimulated THP-1 cells were incubated for 1 h with bucillamine and NAC. Treatment with bucillamine and NAC induced a dose-dependent loss of NF-κB activity in LPS-stimulated THP-1 cells. The bottom band is the unbound probe.

but not by a 50-fold excess of unrelated probe (data not shown). To confirm the specificity of these bands, we performed supershift assays using anti-p50 and anti-p65 antibodies. The addition of anti-p65 or anti-p50 antibody caused a supershift of the bands (data not shown).

Effects of bucillamine and NAC on NF-κB binding to probe in THP-1 cells

The effects of bucillamine and NAC on the direct binding of NF-κB to the probe are shown in Fig. 1. Both drugs inhibited the direct binding of nuclear extracts from LPS-stimulated THP-1 cells in a dose-dependent manner.

Table 2. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on cytokine production from lipopolysaccharide (LPS)-stimulated J774.1 cells

Drugs	Concentration (M)	TNF-α (% inhibition)	IL-6 (% inhibition)
Bucillamine	10^{-5}	−0·9	−31·3**
	10^{-4}	21·8*	5·1
	10^{-3}	58·8**	38·0**
	10^{-2}	90·5**	83·8**
NAC	10^{-4}	9·7	−26·5**
	10^{-3}	42·7**	−2·1
	10^{-2}	72·3**	48·6**

Values were obtained from three to four samples.
*,**Statistically significant compared with the control group (Dunnett's multiple comparison test) (*$P<0·05$; **$P<0·01$).

Effects of bucillamine and NAC on cytokine production in LPS-stimulated THP-1 cells and J774.1 cells

We examined the effects of bucillamine and NAC on the production of TNF-α, IL-1β, IL-6 and IL-8 in the supernatant of each cell culture stimulated with LPS. As shown in Tables 1 and 2, bucillamine and NAC inhibited the production of cytokines in both cell lines in a dose-dependent manner. In addition, bucillamine and NAC also inhibited the TNF-α gene expression at a concentration of 3 mM without inhibition of GAPDH gene expression (Fig. 2). Bucillamine and NAC at a concentration of 10^{-2} M did not induce

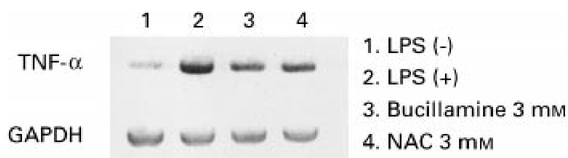

Fig. 2. Inhibitory effects of bucillamine and N-acetyl-L-cysteine (NAC) on tumour necrosis factor-alpha (TNF-α) mRNA expression induced by lipopolysaccharide (LPS). Lanes 1 and 2, TNF-α mRNA of THP-1 cells incubated with or without LPS; lanes 3 and 4, TNF-α mRNA of LPS-stimulated THP-1 cells incubated for 1 h with bucillamine 3 mM and NAC 3 mM, respectively. Bucillamine and NAC inhibited TNF-α mRNA expression in LPS-stimulated THP-1 cells.

Table 1. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on cytokine production from lipopolysaccharide (LPS)-stimulated THP-1 cells

Drugs	Concentration (M)	TNF-α (% inhibition)	IL-1β (% inhibition)	IL-8 (% inhibition)
Bucillamine	10^{-5}	15·3	29·6	−5·6
	10^{-4}	21·2	30·5*	−12·0
	10^{-3}	46·2**	66·1**	28·1**
	10^{-2}	97·4**	88·0**	97·0**
NAC	10^{-4}	19·9	39·9**	−5·3
	10^{-3}	40·1**	47·1**	6·6
	10^{-2}	50·6**	65·0**	63·1**

Values were obtained from three to four samples.
*,**Statistically significant compared with the control group (Dunnett's multiple comparison test) (*$P<0·05$; **$P<0·01$).

Table 3. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on cytokine production in mice

Drugs	Dose (mg/kg)	TNF-α (% inhibition)	IL-6 (% inhibition)
Bucillamine	100	7·0	–
	200	21·9	–
	500	65·7**	16·5*
NAC	100	20·7	–
	200	18·3	–
	500	52·3**	11·3

Values were obtained from four to five animals.

*,**Statistically significant compared with the control group (Dunnett's multiple comparison test) (*$P < 0.05$; **$P < 0.01$).

cell death and apoptosis at least for 4 h incubation assayed by propidium iodine and Annexin V staining using a commercial kit (Genzyme, Cambridge, MA) (data not shown).

Effects of bucillamine and NAC on TNF-α and IL-6 production in mice

LPS stimulation resulted in a plasma TNF-α level of almost 2·8 ng/ml 1 h after injection and a plasma IL-6 level of almost 69·0 ng/ml 2 h after injection. Treatment with bucillamine and NAC at 500 mg/kg resulted in inhibition of TNF-α production. In addition, bucillamine administered at a similar dose (500 mg/kg) inhibited IL-6 production (Table 3).

Effects of bucillamine and NAC on CII-induced arthritis

Immunization of DBA/1 J mice with bovine CII resulted in polyarthritis in almost all animals after secondary immunization. However, treatment with bucillamine and NAC at 500 mg/kg per day resulted in a reduction in the severity of arthritis (Fig. 3). Moreover, bucillamine inhibited bone changes (Fig. 4). Although bucillamine or NAC caused a slight decrease in serum anti-CII antibody level and IL-6 concentration, these effects were not significant (Table 4). There was no significant correlation in individual animals between the severity of arthritis and the degree of decrease in anti-CII antibody level and IL-6 concentration. However, there was a significant correlation between the severity of arthritis and bone changes (correlation coefficient = 0·754). In all animals, no TNF-α was detected in the plasma and no severe drug toxicity effects were observed. Subacute toxicity studies of bucillamine administered at 500 mg/kg per day were previously carried out for a month in male Wistar rats [25]. The haematological examination revealed a slight decrease in erythrocytes, Ht and Hb, and biochemical examination showed a decrease in serum cholesterol levels, phospholipid and triglyceride. However, no body weight loss and no histopathological changes were observed.

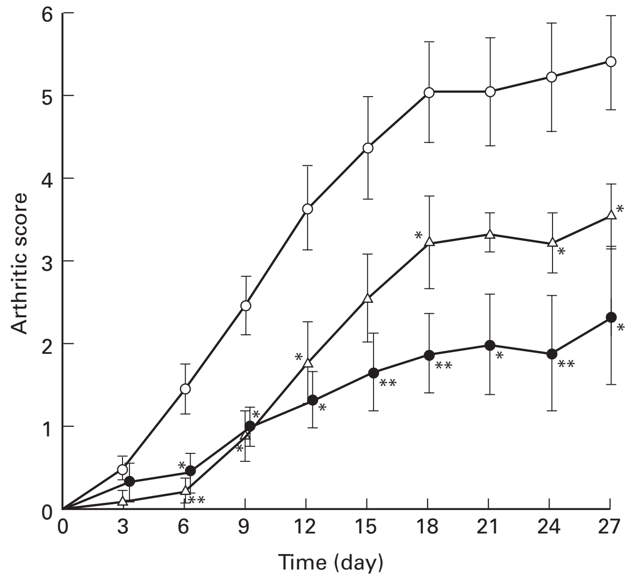

Fig. 3. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on polyarthritis in type II collagen-induced arthritis in mice. Drugs were administered every day from the day of the second immunization. The degree of arthritis was observed every 3 days from the second immunization. Values are expressed as means ± s.e.m. of 9–22 animals. *$P < 0.05$; **$P < 0.01$ *versus* control group by Wilcoxon test. ○, Control; ●, bucillamine 500 mg/kg per day; △, NAC 500 mg/kg per day.

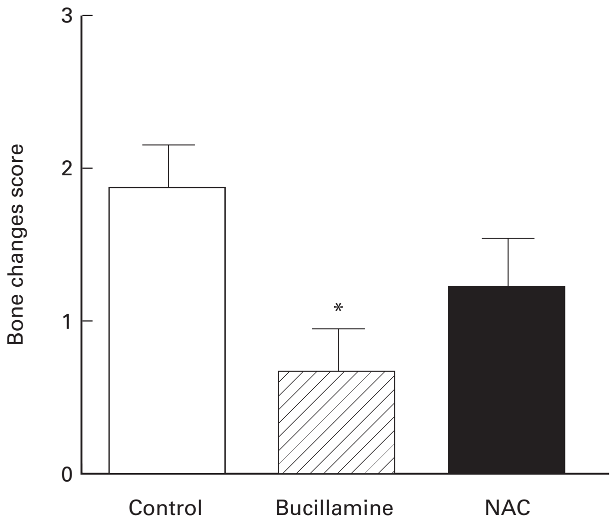

Fig. 4. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on bone changes in type II collagen-induced arthritis in mice. Drugs were administered every day from the day of the second immunization. The degree of arthritis was observed every 3 days from the second immunization. Values are expressed as means ± s.e.m. of 9–22 animals. *$P < 0.05$ *versus* control group by Wilcoxon test. Bucillamine 500 mg/kg per day. NAC 500 mg/kg per day.

Table 4. Effects of bucillamine and N-acetyl-L-cysteine (NAC) on type II collagen (CII) antibody level and IL-6 concentration in arthritic mice

Drugs	Dose (mg/kg per day)	CII antibody level (U/ml)	IL-6 concentration (pg/ml)
Control		286 273 ± 18 572	64·4 ± 15·1
Bucillamine	500	234 201 ± 22 943	25·2 ± 10·6
NAC	500	244 281 ± 32 757	32·5 ± 18·5

Values were obtained from nine to 22 animals.

DISCUSSION

To investigate the role of NF-κB in cytokine production and CIA, we used two NF-κB inhibitors, bucillamine and NAC. We demonstrated that bucillamine and NAC inhibited NF-κB activation and TNF-α mRNA expression in human monocytic leukaemia cell line THP-1, and cytokine production from monocyte cell lines at concentrations above 10^{-3} M. These findings may indicate a possible correlation between the inhibition of NF-κB activation, the inhibition of TNF-α mRNA expression and the inhibition of cytokine production *in vitro*. They also inhibited cytokine production and CIA in mice at a dose of 500 mg/kg. Bucillamine and NAC inhibited TNF-α production more strongly than IL-6 production both *in vitro* and *in vivo*. In addition, bucillamine exhibited somewhat stronger effects than NAC both *in vitro* and *in vivo*. There was no significant correlation in individual animals between the severity of arthritis and the degree of decrease in anti-CII antibody level and IL-6 concentration. However, the degree of inhibition of the severity of arthritis by treatment with bucillamine and NAC paralleled the degrees of decrease in anti-CII antibody level and IL-6 concentration.

With some encouraging preliminary data [26–28], NAC has been proposed as an agent for treatment of human sepsis and adult respiratory distress syndrome (ARDS). Animal studies have shown that NAC reduces endotoxin-induced neutrophil activation in sheep [29], protects against phosgene-induced lung injury in rabbits [30], and diminishes paraquat- and IL-1-induced neutrophilic lung inflammation in rats [31,32]. Blackwell *et al.* [33] reported that NAC probably blocks neutrophilic inflammation in part by diminishing the NF-κB-dependent transcription of the cytokine-induced neutrophil chemoattractant (CINC) gene in rat lung inflammation models. They showed that treatment with NAC (200–1000 mg/kg) dose-dependently decreased lung NF-κB activation. Blocking NF-κB activation may also reduce the transcription of a variety of other genes involved in causing inflammation. The report suggests that the dose of bucillamine or NAC used in our study may be sufficient for inhibition of NF-κB activation *in vivo*.

Sha *et al.* [34] reported that targeted disruption of the p50 subunit of NF-κB led to multifocal defects in immune responses involving B lymphocytes and non-specific responses to infection. Recent advances in our knowledge of the function and chemistry of proteins involved in gene expression have indicated that thiol groups in the proinflammatory transcription factors AP-1 and NF-κB are targets for at least some of the therapeutic effects of DMARD [21]. Developments in understanding the transcriptional effects of glucocorticoid and retinoid receptors have indicated that they too act, at least in part, via inhibition of AP-1 and/or NF-κB activities. Fujisawa *et al.* [35] reported that suppression of NF-κB could be a potential therapeutic modality for synovitis such as that of RA. Our results using two NF-κB inhibitors are consistent with the involvement of NF-κB activation in RA.

In our study, bucillamine exhibited somewhat stronger inhibitory activity against NF-κB activation than NAC. Aono *et al.* [36] also reported that the proliferation of human synovial cells and IL-1β and IL-6 production of human synovial cells were significantly inhibited by bucillamine. Activation of NF-κB is involved in not only cytokine production but also synovial cell proliferation [35]. Although further investigations are necessary to make clear the clinical effects of bucillamine, the inhibition of NF-κB activation may be one of the anti-rheumatic mechanisms of bucillamine similarly caused by glucocorticoids, gold, retinoids and penicillamine. It should also be noted that in addition to its possible use in RA, bucillamine may be useful for treatment of human sepsis and ARDS.

In conclusion, NF-κB inhibitors such as bucillamine and NAC may inhibit cytokine-related diseases including arthritis.

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